Scanning Microscopy

Volume 1994
Number 8 *The Science of Biological Microanalysis*

Article 8

6-3-1994

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Scanning Microscopy Supplement 8, 1994 (pages 101-108) 0892-953X/94$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

QUANTITATIVE BIOLOGICAL ELECTRON PROBE MICROANALYSIS WITH A WAVELENGTH DISPERSIVE SPECTROMETER

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(Received for publication September 29, 1993, and in revised form June 3, 1994)

Abstract

This paper describes the details of quantitative electron probe microanalysis (EPMA) performed with a wavelength dispersive spectrometer (WDS). EPMA was carried out on the giant neuron of a fresh frozen ganglion from the snail *Lymnaea stagnalis*. The freeze-dried cryosections were compared with sections of freeze-dried, embedded tissue. It was found, that in the ganglion there are two kinds of neurons with a different chlorine concentration of 11 mmole/liter and 32 mmole/liter. Isolated neurons in culture were shown to differ in elemental composition from those in the ganglion tissue.

Key Words: Biological electron probe microanalysis, wavelength dispersive spectrometer, quantitation, cryopreparation, sensitivity, resolution, snail giant neuron.

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Introduction

Electron probe microanalysis (EPMA) allows the study of the elemental distribution and composition in tissues. The retention of the elemental content in soft biological tissues is one of the problems of preparation for EPMA. Elements may be retained by precipitation using a histo- or cytochemical reaction (Komnick, 1962; Engel, 1968; Tandler *et al.*, 1970; Rosen and Beeuwkes, 1979; Wood, 1979; Przelecka and Pogorelov, 1988). A more general approach is the fixation of labile elements by freezing the soft biological tissues (Costello and Corless, 1978). There are several ways to prepare the tissue following the cryofixation (Roomans *et al.*, 1982; Echlin, 1992), which allow the frozen samples to be analyzed in the hydrated and dried state.

Application of EPMA also is dependent on the sensitivity of the method. It is required that the elemental concentration in the specimen is higher than the minimal detectable concentration (MDC). This parameter can be improved by the choice of the cryotechnique. Therefore, even if the preparative procedure fulfills the experimental requirements (such as morphological resolution, and retention of the *in vivo* distribution of elemental content and water), also the sensitivity of the analysis should be taken into account.

Most groups engaged in biological EPMA apply an energy dispersive spectrometer (EDS) technique and cryosectioning as the preparative method for the analysis of the diffusible elements in soft biological tissue. Many papers discuss the achievements in the quantitative analysis of dried and hydrated samples by EDS. However, much less is known about the application of wavelength dispersive spectrometry (WDS) to EPMA. The present paper aims to summarize the experience (preparation and quantitation) in biological EPMA carried out by WDS.

Materials and Methods

Resolution and sensitivity

It was shown (Pogorelov, 1987) that the resolution of EPMA in bulk specimens and thick sections (i.e., section thickness more than the electron beam can penetrate) could be calculated from:

$$r(E) = 2.86 * E^{2.29} * 10^4 \quad (1)$$

with the lateral spatial resolution r(E) expressed in units of the mass thickness ($\mu m*g/cm^3$) and the accelerating voltage (E) in MeV. According to eqn. (1) the value of r(E) is equal to 6 $\mu m*g/cm^3$ at 25 kV. The effective depth of electron penetration (R) in the thick section (bulk specimen) is calculated from:

$$R(E) = 0.464 * E^{1.66} * 10^4 \quad (2)$$

with the effective depth R(E) expressed in units of the mass thickness $\mu m*g/cm^3$ at 25 kV.

The section is considered as thin one as long as the rate of X-ray counts is linearly related to the section thickness. At an accelerating voltage of 25 kV this requirement is fulfilled up to about 6 $\mu m*g/cm^3$ (Pogorelov and Allachverdov, 1984). The morphological resolution in thin sections was demonstrated by Burovina *et al.* (1978) to be 0.5 μm or better.

The sensitivity of analysis in thin sections can be calculated according to eqn. (3) (Pogorelov, 1987):

$$MDC(l) = K * \sqrt{[d/(i*t*l)]} \quad (3)$$

with MDC(l) as the sensitivity of EPMA for a section thickness l; K is the proportionality coefficient; d is the section density; i is the beam current; t is the time of analysis. The dependence between the values of MDC for bulk specimens and MDC(l) for thin sections prepared from the same tissue is described by:

$$MDC = MDC(l) * \sqrt{[k(A)*l*d]} \quad (4)$$

where k(A) is the X-ray intensity ratio for element "A" in a thin section (I_{sec}) and in a bulk specimen (I_{bulk}), respectively, as determined from:

$$I_{sec}/I_{bulk} = k(A)*l*d \quad (5)$$

with the same abbreviations as in eqn. (3). For a thick section the ratio in eqn. (5) is equal to 1. The value of the coefficients in eqns. (3) and (5) was measured with a biological standard similar in chemical composition and density to biological tissue, and with known concentrations of elements in the physiological range (Roomans and Sevéus, 1977; Saubermann *et al.*, 1981a). The MDC for biological EPMA carried out by WDS is presented in Table 1.

Section preparation

Fresh ganglia from the snail *Lymnaea stagnalis* were cryofixed by plunging in liquid propane cooled to 90 K with liquid nitrogen. Specimens were prepared according to the methods described for the study of diffusible elements in soft biological tissues (Ingram *et al.*, 1972; Dörge *et al.*, 1978). Frozen specimens were freeze-dried in high vacuum at low temperature. The freeze-drying procedure employed has been described by Pogorelov *et al.*, (1991). After freeze-drying at 163 K, and at a pressure of $5*10^{-4}$ Pa the tissue was warmed, and then embedded in epoxy resin. 2-μm thick sections were cut using a dry glass knife at room temperature in a Reichert ultramicrotome.

Some frozen specimens were transferred into a Sorvall Porter-Blum MT2 ultramicrotome (Christensen, 1971) equipped with a modified cryochamber (Saubermann *et al.*, 1981b; Biddlecombe *et al.*, 1982). Cryosections of frozen tissue, 2 μm thick, were cut at 223 K, one at a time with a steel knife with an angle of 40°, set at 5° clearance angle. Cryosections were picked up from the knife edge with an eyelash probe, placed between collodium films and freeze-dried at 163 K for 5 hours at $5x10^{-4}$ Pa. The sections of freeze-dried, embedded tissue and freeze-dried cryosections were mounted on Cu grids without supporting films, coated in a Micro BA 3 (Balzers) vacuum unit with a conductive carbon layer approximately 10 nm thick.

The samples were examined in a JEOL JSM-U3 scanning electron microscope. This microscope is equipped with two wavelength dispersive spectrometers (WDS) with a take off angle of 30°. A mineralogical polished standard was used to control the WDS adjustment, since such standards are stable and generate a high X-ray count rate (Table 2). Thin sections were viewed in the transmitted electron mode using a dark field detector, at an accelerating voltage of 25 kV. The probe diameter was about 0.1 μm. The inner and outer half angles of the darkfield detector were 10 and 55 mrad. The bulk specimens were observed in the secondary electron mode.

Quantitation

The well-known Hall procedure (Hall, 1971; Hall and Gupta, 1979) is not usually employed in WDS because of the low intensity of the continuum count registered with a WDS. The quantitative method used here is based on the calibration of characteristic X-ray

Table 1. Minimal Detectable Concentration (mmole/liter) for EPMA of dried soft biological tissue sections.

Sample Analyzed	Element Analyzed				
	calcium	potassium	chlorine	sodium	phosphorus
1 μm section	6	8	10	20	20
Thick section	1.0	1.3	1.7	5.8	3.4

The data were calculated for a JSM-U3 (JEOL) microanalyser at an accelerating voltage of 25 kV. The beam current was 20 nA, the time of analysis was 40 seconds and the sample density was 0.20 g/cm^3.

Table 2. Rates of the Kα line (counts/nA sec) of different elements on mineralogical and bulk dextran standards

standard used	Element Analyzed							
	Na	Si	Al	Ca	K	Cl	S	P
NaCl	68	---	---	---	---	300	---	---
$KAlSi_3O_8$	---	490	58	---	165	---	---	---
$Ca_3(PO_4)_2$*$2H_2O$	---	---	---	690	---	---	---	62
Fe_2S	---	---	---	---	---	---	98	---
20 % dextran*	1	---	---	3	22	8	5	3

The X-ray count rate was measured with the crystals RAP (Na, Al, Si) and PET at an accelerating voltage of 25 kV.
* The concentration of the analyzed elements in the dextran standard was 0.4 mole/kg dry wt (corresponding to 0.1 mole/liter) except for calcium, where the concentration was 0.04 mole/kg dry wt (corresponding to 0.01 mole/liter)

intensity (Pogorelov and Allachverdov, 1984). The element concentration C(A) in the thin tissue sections was calculated from:

$$C(A) = (I_{sec} * K(A))/(I_{st} * k(A) * l) \qquad (6)$$

where I_{sec} and I_{st} are the X-ray intensities of element "A" in the thin section or mineralogical standard, respectively; k(A) is the coefficient described in eqn. (5); the coefficient K(A) describes the difference between chemical composition of the massive mineralogical standard and soft biological tissues. The equation above is correct at 25 kV if the maximal value of mass thickness of the thin section is 6 μm*g/cm^3 and 3 μm*g/cm^3 for the Kα line of K (Ca,Cl,S,P) and Na, respectively.

The elemental concentration calculated is expressed in terms of mole/liter for the thin sections. For the thick section (bulk specimen) the value of (k*l) is transformed to 1 and the element concentration is expressed in terms of weight fraction (mole/kg weight of the analyzed sample).

The coefficient k(A) was calculated using eqn. (5) on the basis of data obtained experimentally with a standard consisting of sections of 20% dextran solution. These standards were prepared from 2 μl droplets according to the cryomethods described above. The dextran standard was homogenous (Fig. 1) and contained physiological concentrations of the elements analyzed. The coefficient K(A) in eqn. (6) was shown to be dependent on the density of the porous samples for elements with atomic number less than 12 (Ichinokawa *et al.*, 1969; Pogorelov, 1987). This is a limiting factor for quantitative EPMA of soft biological tissue.

Results and Discussion

Analysis of thin sections

The fraction of the incident current transmitted through the thin section is sufficiently high to use the scanning transmission electron microscopy (STEM) mode (Cosslet and Thomas, 1966). It was shown by Pogorelov *et al.* (1991) that for 2-μm sections of unstained tissue at 25 kV the resolution in the STEM mode is comparable to light microscopy and the contrast is better than that for secondary electrons (Fig. 2). The good spatial resolution and contrast in the 2 μm thick section are consistent with the theory for thin films (Beaman, 1979; Edie and Karlsson, 1972; Missel and Burdett, 1977).

Elemental concentrations close to physiological

values can be measured in sections thicker than 1 μm (Table 1). Eqn. (3) shows that the sensitivity of analysis can be improved not only by using freeze-dried cryosections with low density but also by increasing the probe current. However, a beam current higher than 4 nA results in damage to the dried cryosections.

Sections of freeze-dried, embedded tissue are more stable and allow the beam current to be increased to about 25 nA. Unfortunately, that increase does not improve the MDS(l) significantly because of the higher density of embedded tissue as compared with the dried cryosection (the average values correspond to 1.20 and 0.20 g/cm^3, respectively).

The quantitative data obtained on the thin sections of ganglia from *Lymnaea stagnalis* are presented in Table 3. The section thickness is the main factor determining the quantitation according to eqn. (6). We found that at room temperature the thickness of the thin sections was not dependent on the epoxy resin used for embedding (Epon, Spurr, Araldite, Lowicryl) provided that a mechanically stable microtome (Reichert or Porter Blum MT2) was used. The thickness of cryosections depends more critically on the experimental conditions as, for instance, temperature of sample and knife, cutting speed, knife material, edge angle. (Roomans *et al.*, 1982). This dependency results in more variation in the thickness of cryosections which explains the higher standard deviation observed in freeze-dried cryosections (Table 3).

The disadvantage of the embedded tissue is that almost all the widely used epoxy resins (Epon, Spurr, Araldite) contain high concentrations of chlorine, which does not permit this element to be analyzed in sections of resin-embedded specimens. However, this problem can be avoided using the chlorine free Lowicryl K4M resin (Pogorelov *et al.*, 1991). An important advantage of the embedded tissue is, that it can easily be stored for a long time (years). Moreover, structures with low density (such as cell walls, contents of lymph capillaries or lumen, mucus layers) are well protected from the mechanical damage during sectioning and the procedure following that. Embedded samples provide a useful opportunity for the study of the cellular composition by other methods of cytology (light microscopy, autoradiography, cytochemistry, fluoroscent microscopy etc).

Analysis of thick sections

Eqn (4) shows that the best MDC can be obtained for freeze-dried thick sections (bulk specimens). Higher sensitivity is especially important for the EPMA of calcium and sodium. Therefore, freeze-dried pieces of ganglia were analyzed as bulk specimens. The surface which was freeze-fractured by cryosectioning

Fig 1. Scanning transmission electron image of the surface of freeze-dried cryosections, 2 μm thick, of dextran (left) and albumin standards. Scale bars = 2 μm.

Fig 2. Analysis of freeze-dried cryosections, 2 μm thick, of giant neuron from a ganglion of the snail *Lymnaea Stagnalis*. The scanning transmission electron image (a) and the corresponding X-ray map of potassium (b); c= cytoplasm, n= nucleus, g= glial tissue. Scale bars = 10 μm.

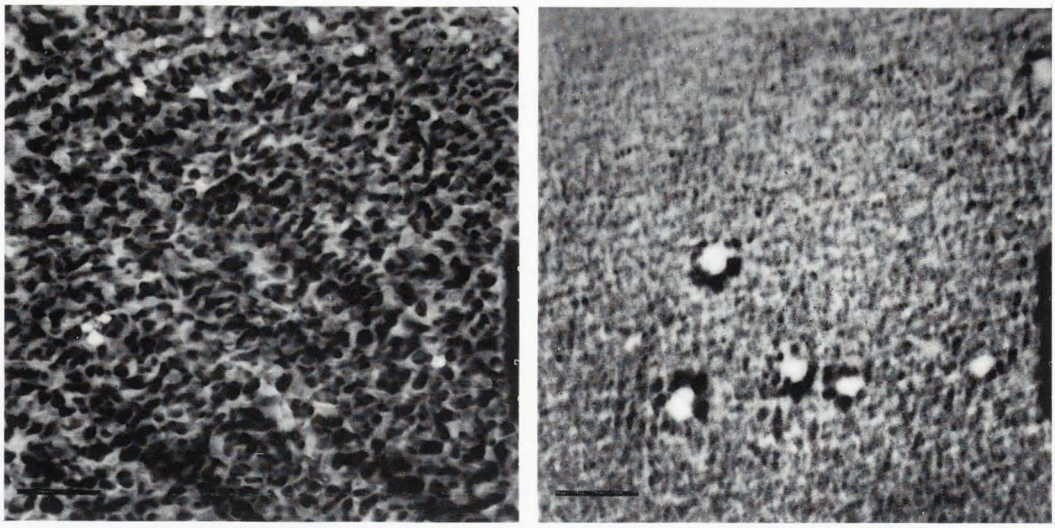
Fig 3. Secondary electron image of the surface of a freeze-dried ganglion freeze-fractured by cryosectioning. Ganglion of the snail *Lymnaea stagnalis* (a) and neuron (b); c= cytoplasm, n= nucleus, g= glial tissue. Scale bars = 20 μm.

was observed in the secondary electron mode (Fig. 3). The resolution of EPMA for bulk samples was determined by the size of the zone ionized by the electron beam.

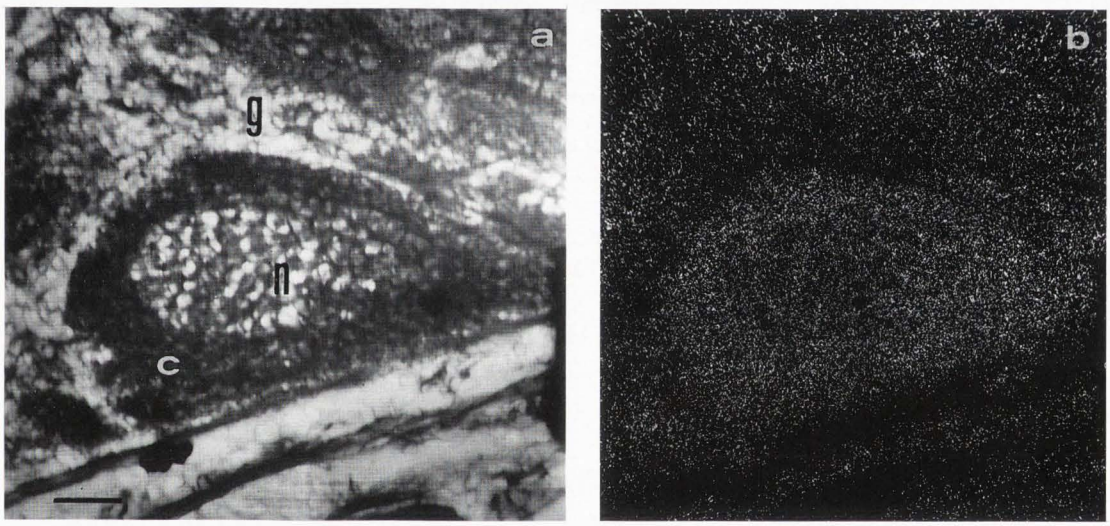
According to eqn. (1), at an accelerating voltage of 25 kV in dried tissue with a density of 0.2 g/cm^3 the lateral resolution and the effective depth of beam penetration are equal to 30 μm and 60 μm, respectively. This means that the analyzed volume can be much larger than that of a neuron imaged in the secondary electron mode. Hence, the data obtained for bulk specimens reflect the average element concentrations in the part of tissue from the surface down to the depth of electron penetration. This averaging explains that the two types of neurons identified with chloride-sensitive microelectrodes (Kerkut and Meech, 1966) in thin freeze-dried cryosections according to their Cl concentration (Table 3) were not found in the bulk specimens of the ganglia (Table 4). The concentrations of other elements (K, Na, Ca, P) were in good accord with those measured in thin sections.

Also a single cultured cell can be analyzed by EPMA as a bulk sample. In the present study, we examined a primary culture of giant neurons of the snail *Lymnaea stagnalis* (Fig 4). The cells were cultured on a glass support and prepared as published earlier by Kostenco *et al.* (1983). After incubation for 1 day the neuron culture (not washed) on a glass support was frozen in liquid propane and freeze-dried. To avoid contamination of the spectra by elements of the support an accelerating voltage of 15 kV was chosen. The map of silicon distribution confirms that Si, the main element found in the glass support was not detected below the cells.

The quantitative data obtained for the isolated



1



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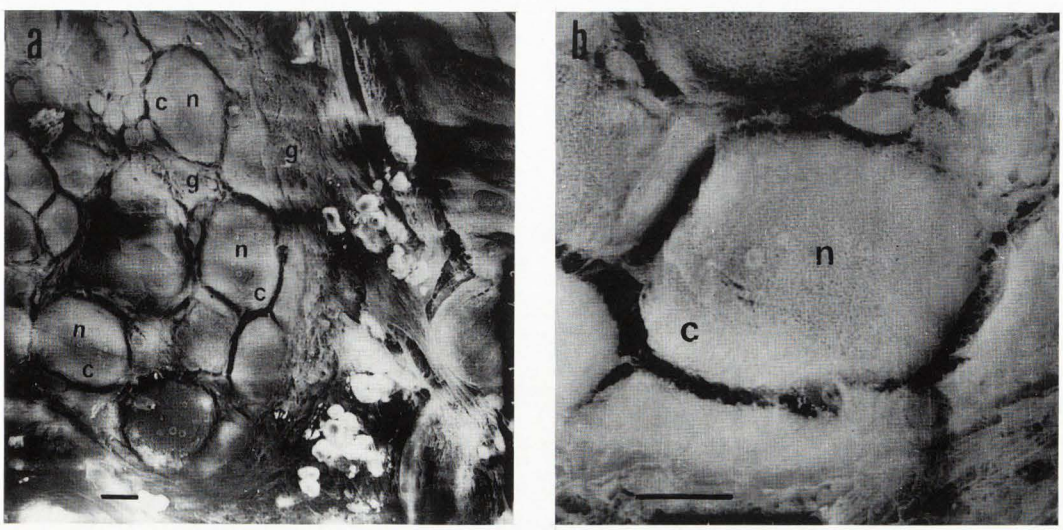

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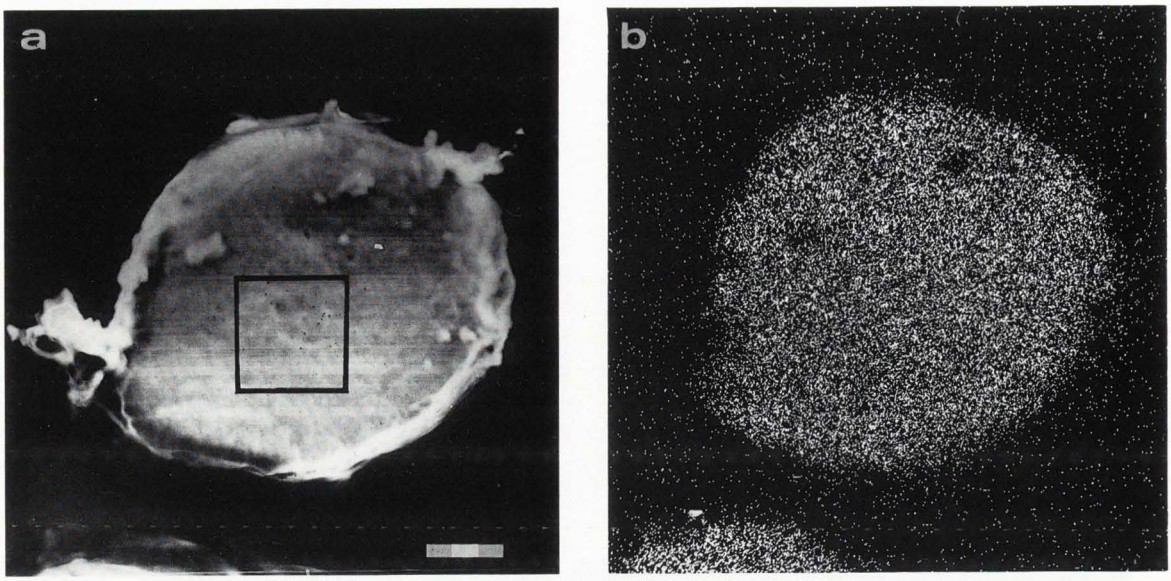

Fig 4. Analysis of a freeze-dried neuron isolated from ganglion of the snail *Lymnaea stagnalis* and incubated for 1 day on a glass support. Secondary electron image of the single neuron (a) and corresponding distribution of potassium (b). The analysis was carried out within the square marked. Scale bars = 10 μm.

neurons are presented in Table 4. The elemental concentrations in this sample are dramatically different from those measured in thin sections (Table 3) and bulk specimens (Table 4) of ganglion tissue. Elevated amounts of Na and Ca were found in the isolated neurons. Concentrations of K and P were decreased, but Cl was the same as in the bulk specimen.

These changes were not surprising. It was shown by Kendall *et al.* (1985) that isolated cells differed in elemental composition from those in the corresponding tissue. Evidently, the increase of Na and Ca, and the loss of K in the isolated neurons reflect an adaptive reaction of cell to the changed conditions.

The data obtained allow us to conclude that EPMA with WDS provides a method for the analysis of physiological elements close to their *in vivo* concentrations. The resolution of EPMA can be significantly improved by the choice of techniques of specimen preparation. Cryotechniques can be applied not only for the retention of labile elemental contents in soft biological tissues, but also as a way to optimize the conditions of EPMA.

Acknowledgements

We wish to express our thanks to Dr Thomas von Zglinicki and Dr E. Vulfius for valuable comments on this paper, and Dr N. Burmostrova for excellent assistance with cryosectioning. This work was supported by a International Science Foundation grant No NYD 000 and by the European Microbeam Analysis Society (EMAS).

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Table 3. Elemental concentration (mmole/liter) in the cytoplasm of giant neuron from the ganglia of the snail *Lymnaea stagnalis* (mean ± standard deviation)

Preparation Method	Element Analyzed potassium	sodium	calcium	phosphorus	chlorine
freeze-dried	125±30	27±8	n.d.*	n.m.*	11±5 (15)
cryosection	number of neurons measured (68)				32±6 (53)
section of	112±6	25±5	n.d	220±20	n.d.
FDE tissue	number of neurons measured (21)				

Data were obtained on either 2 μm thick freeze-dried cryosections, or on sections of freeze-dried, embedded (FDE) tissue at an accelerating voltage of 25 kV

* n.m.- not measured, n.d.- not detected, in parentheses the number of neurons measured

Table 4. Elemental content (mmole/kg dry weight) measured in the neuron ganglia from *Lymnaea stagnalis* using bulk tissue specimens and isolated cells (mean ± standard deviation)

Sample Analyzed	Element Analyzed potassium	sodium	calcium	chlorine	phosphorus
freeze-dried	600±30	160± +20	20±6	180±30	800±100
bulk sample	(120)	(31)	(4)	(36)	(160)
freeze-dried	260±20	300±40	70±20	150±20	70±10
isolated neuron	(52)	(60)	(14)	(30)	(14)

Accelerating voltages of 25 kV and 15 kV were used for the freeze-dried bulk samples and the isolated samples, respectively. The number of neurons measured on the freeze-dried bulk specimen was 25, and that for isolated neurons was 18. In parentheses the elemental concentration recalculated as mmole/liter, based on the assumption that the average density of the dried neuron is 0.2 g/cm^3.

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Editor's note: All questions by reviewers were answered by text changes. There is hence no Discussion with Reviewers.